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Award Number: W81XWH-10-0567

TITLE: A long stress-responsive non-coding transcript (NiT 5) and its role in the development of breast cancer

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REPORT DATE: August 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2012	Annual	1 August 2011 – 31 July 2012

4. TITLE AND SUBTITLE

A long stress-responsive non-coding transcript (NiT 5) and its role in the development of breast cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-0567

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Mayo Clinic
Rochester, MN 55905

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this idea award was to characterize one novel long non-coding transcript that we had identified in a screen using whole-genome tiling arrays. This transcript which is called Long Stress-Induced Non-Coding Transcript 5 (LSINCT5) was one of 12 long non-coding transcripts that we had identified. This transcript was chosen over the other 11, as it had increased expression in most of the breast cancer cell lines and primary tumors that we analyzed. There were three Specific Aims to our original proposal aimed at characterizing this transcript and determining what role it played in the development of breast cancer. While we have been successful in completing most of our original Specific Aims we have struggled in the past year because the level of expression of this transcript is so low. This has made the characterization of this transcript difficult, but we have now been able to reproducibly measure this transcript. In the final year of this Idea Award we propose to finish up the remaining work from our original Specific Aims, but also have several additional things we'd like to do in order that we can successfully compete for extramural funding from the National Institutes of Health.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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Introduction

The goal of this work is to characterize a novel long non-coding transcript. This transcript was one of 12 stress-induced long non-coding transcripts that we identified. We chose to study this one transcript, as compared to the other eleven because it had increased expression in most breast cancer cell lines and primary breast cancers that we analyzed. However, the level of expression of this long non-coding transcript is quite low and this has posed a considerable problem for us, in terms of reproducibly measuring this transcript. We had three original Specific Aims (seen on the next page), and we have already completed much of the proposed work. This year we have had considerable difficulty measuring this very low expressed transcripts and indeed the first postdoctoral fellow left the laboratory after failing to measure this transcript. A second postdoctoral fellow is now working on this, but she has just now (after almost 8 months of work) been able to reproducibly measure this transcript. We therefore have little in the way of additional data (above and beyond what we described in our last years' annual report). The goal in the next year is to finish up a few experiments from our original Specific Aims (mainly to determine if LSINCT5 does co-localize with another long non-coding transcript, NEAT1, and within the nuclear paraspeckles). In addition, we are proposing several additional experiments whose aim is to determine what other transcripts and/or proteins that interact with LSINCT5, and some studies to determine which cellular stress pathways LSINCT5 is involved with. These studies are important in order for us to attempt to compete for extramural funding from the NIH to continue this work.

BODY

ORIGINAL SPECIFIC AIMS

Specific Aim #1: Characterization of LSINCT5
We will first more fully characterize LSINCT5 (formally called NIT5). We will determine where this transcript begins and ends using 5' and 3' RACE as well as RNase protection assays. We will also characterize which polymerase is involved in its transcription. Lastly, we will identify where the LSINCt5 transcript resides in both normal and breast cancer cells.

Specific Aim #2: Analyze the phenotypic effect of modulating LINCT5 expression
We will use siRNA technology to decrease the expression of LSINCT5 in breast cancer cell lines where it is most highly over-expresssed. We will also construct a full-length LSINCT5 expression construct to over-express it in normal human breast epithelial cell lines (HMEC and MCF10A). We will also make stable constructs with either increased or decreased expression of LSINCT5. We will then use a variety of different assays including proliferation assays (MTT and BrDU stain), migration assay (Boyden chambers), soft agar assay, and apoptosis assays (Annexin V/PI strain, LIVE/DEAD Fixable Dead Cell strain Kit, and Tunnel Assay) to determine the phenotypic effect of modulating LSINCT5 expression.

Specific Aim #3: To characterize genes, non-coding transcripts and pathways that are affected by the modulation of LSINCT5 expression
We will utilize the power of Next Generation DNA sequencing to characterize the effect of modulating the expression of LSINCT5 on the entire transcriptome of either normal breast epithelial cell lines or breast cancer cell lines. Hence, we will be able to characterize which coding genes are affected by modulating LSINCT5 expression as well as non-coding transcripts that have altered expression as a result of changing LSINCT5 expression. This work will enable us to determine how LSINCT5 is involved in cellular proliferation and which genes and pathways LSINCT5 interacts with.

Results and Progress

The past 18 months have been extremely frustrating in terms of generating additional data on the role that LSINCT5 plays in both normal cells and during breast cancer development. LSINCT5, and the rest of the LSINCTs, was identified by my graduate student Jessica Silva, who did this work as part of her Ph.D. project at the Mayo Clinic. Jessica graduated in the spring of 2010 and is currently working as a post-doctoral fellow in the laboratory of Dr. Elaine Mardis (at Washington University in St. Louis).

Jessica identified and characterized a total of 12 long non-coding stress-induced transcripts. She chose to work on LSINCT5, instead of any other of the long non-coding transcripts that we identified, because this one non-coding transcript had increased expression in most of the breast cancer cell lines and primary tumors that we tested. However, a serious drawback and problem with working with this transcript is that it has very low expression. This is in contrast to several of the other long non-coding transcripts that Jessica had identified and also to two very interesting previously characterized stress-induced non-coding transcripts, NEAT1 and NEAT2. Indeed NEAT1 and NEAT2 are quite abundantly expressed in many different cell types.

The very low expression of LSINCT5 posed many problems for Jessica in terms of detecteing this transcript, and necessitated Jessica to go to extraordinary measures to reliably measure LSINCT5 expression and knock-down. This included using considerably more total RNA when she did cDNA synthesis in order to detect LSINCT5 expression at all. After Jessica left, and with the support from the Department of Defense Breast Cancer Program, I recruited a post-doctoral fellow to continue this work. Unfortunately, the first post-doc that I hired, could never reliably measure LSINCT5 expression and after 6 months of totally negative results, I had no choice but to ask that post-doctoral fellow to leave the laboratory. Last January, I hired a second postdoctoral fellow, but she too has had nothing but trouble measuring LSINCT5 expression. This post-doc spent almost 7 months working out conditions to try to reliably measure LSINCT5 expression, and she now can reliably measure the low expression of LSINCT5. As a direct result of this, we have almost no additional results to report in this annual report. Last spring we requested, and were granted, a one year, no-cost extension and we hope that in the next year to get some additional data about LSINCT5.

Fortunately, Jessica Silva had already completed much of the work in our three Specific Aims. As outlined in last years' report, we have characterized LSINCT5 and found it to be a 2,647 base polyadenylated transcript. This transcript is localized in the nucleus, and it is transcribed off the negative strand by RNA polymerase III. For the second Specific Aim, we usedan allele specific oligonucleotides (since LSINCT5 is a nuclear-encoded transcript, siRNA strategies did not work to knock down LSINCT5 transcript levels) to generate a 50% knock-down of LSINCT5 expression. Any greater knock-down resulted in the cells undergoing apoptosis. We also found that this knock-down resulted in decreased cellular proliferation, which fits our overall hypothesis that the increased expression of LSINCT5 in breast cancers results in the breast cells being more proliferative. We also examined the effect of knocking down LSINCT5 expression on the expression of the other 20,000 protein coding genes, and this led to our observation that two of the genes whose expression went down after knocking down LSINCT5 expression were the long non-coding RNA NEAT1 and the protein coding gene paraspeckles. All of this work was part of a paper which we published in 2011 (see publications). There were a number of other genes that had significantly altered expressed after knock-down of LSINCT5, including several genes that have known roles in cancer development. We published a very nice paper in *RNA Biology* describing the more detailed characterization of LSINCT5 and the observation that its' expression has something to do with cellular proliferation.

The experiments that we wish to start (but that were outside the scope of our original proposal) were to examine the precise localization of LSINCT5 in the nucleus, to determine if LSINCT5 did localize with both NEAT1

and paraspeckle, two of the transcripts that have reduced expression in response to LSINCT5 knock-down. The second set of experiments is to try and determine what protein molecules are associated with the LSINCT5 transcript. Our strategy to do this is to produce a biotin labeled LSINCT5 probe and then to hybridize this to nuclear extracts from MCF7 cells. Any proteins that are pulled down using strepavidin coated magnetic beads will be analyzed by the Mayo Clinic Proteomics Facility using one of their Mass Spectrometers to determine important proteins that might be involved with or interact with the LSINCT5 transcript. These are important questions that need to be answered so that we will be more competitive for extramural funding when we submit our proposal on LSINCT5 to the National Cancer Institute. We did try one round of submitting an RO1 on LSINCT5 to the NCI, but the main reviewer comment (and the reason that this grant was triaged out) was that we still don't know what LSINCT5 does in the cell or which proteins or transcripts it interacts with.

There are a third set of experiments that we would like to conduct with LSINCT5. LSINCT5 was identified as a stress-induced non-coding transcript because it was induced when cells are cultured in the presence of the DNA damaging agent NNK. What we did not know is what other types of cellular stress would induce LSINCT5 expression. We obtained two very interesting cell lines produced from transformed lymphoblasts. The first one, GM1666a, is derived from a patient who has ataxia telangentsia because of a mutation in ATM. The second cell line, GM1667a, was produced from GM1666a, by transducing in a fully functional ATM gene. We can therefore compare different cellular stresses both in the absence and in the presence of a functional ATM gene. We will take a variety of chemicals that induce different types of cellular stress. This will include NNK, Rapamycin, taxol, 17-AAG, and 2-deoxyglucose. By examining which cellular stresses induce LSINCT5 expression (again in the presence and absence of ATM) we hope to get a better understanding of the pathways that regulate LSINCT5 expression.

Conclusions: Thus, in the last year (our no-cost extension year) we will work to determine if LSINCT5 does indeed co-localize with both NEAT1 with the nuclear paraspeckles. We will then do pull-down assays to attempt to determine what proteins (and transcripts) LSINCT5 is associated with. Finally, we will begin to characterize the precise pathways of cellular stress that induce LSINCT5 expression.

References

Publications Resulting from this Work

Silva JM, Perez DS, Pritchett JR, Halling ML, Tang H, and **Smith DI.** Identification of long stress-induced non-coding transcripts that have altered expression in cancer. *Genomics* 2010; 95(6): 355-362.

Silva JM, Boczek NJ, Berres MW, Ma X, and **Smith DI.** LSINCT5 is over-expressed in breast and ovarian cancers and affects cellular proliferation. *RNA Biol* 2011; 8(3): 496-505.

Abstracts Resulting from this Work in the past year.

LSINCT5 is over-expressed in breast and ovarian cancers and affects cellular proliferation. Presented at the Non-coding RNA and Cancer meeting. Eden Roc Resort, Miami Florida, Jan. 10, 2012.